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Research Article

Mechanism of Carvedilol-Induced Block of Delayed Rectifier K^+ Current in the NG108-15 Neuronal Cell Line

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Strategy, Management and Health Policy				
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ABSTRACT The effects of the β-adrenoceptor antagonist carvedilol on delayed rectifier K^+ current ($I_{K\,(DR)}$) were examined in NG108-15 neuronal cells. Carvedilol (1–100μM) reversibly blocked $I_{K\,(DR)}$ with an IC_{50} value of 5 μM. $I_{K\,(DR)}$ in response to depolarizing pulses was sensitive to inhibition by quinidine or dendrotoxin, but not by iberiotoxin, 5-hydroxydecanoate sodium, or linopiridine. The carvedilol-induced inhibition of $I_{K\,(DR)}$ could not be reversed by further application of *t*-butyl hydroperoxide or diazoxide. The inhibition of $I_{K\,(DR)}$ by carvedilol could still be observed in cells preincubated with *t*-butyl hydroperoxide (1 mM), ruthenium red (30 μM), or carbonyl cyanide *m*-chlorophenyl hydrazone (10 μM). The presence of carvedilol enhanced both the rate and extent of $I_{K\,(DR)}$ inactivation. Recovery from block by carvedilol (3 μM) could be fitted by a single exponential with a value of 1.64 s. Crossover of tail currents in the presence of carvedilol was also observed. Cell-attached single-channel recordings revealed that carvedilol suppressed channel activity without altering single-channel amplitude. With the aid of the binding scheme, a quantitative description of the carvedilol actions on $I_{K\,(DR)}$ was also developed that clearly showed that in addition to being an antioxidative agent, carvedilol can block delayed rectifying K^+ channel of neurons in an open- and state-dependent manner. Drug Dev. Res. 58:196–208, 2003.

Key words: carvedilol; delayed rectifier K^+ current; NG108-15 cells

INTRODUCTION

Carvedilol is a nonselective β-adrenoceptor blocker with free radical scavenging activity [Lysko et al., 1992; Tadolini and Franconi, 1998] that has a number of potentially beneficial pharmacological effects including antioxidant and antiproliferative activities [Ohlstein et al., 1993; Moser and Frishman, 1998; Tadolini and Franconi, 1998; Abreu et al., 2000; Dandona et al., 2000]. Because of the lipophilic nature of this compound, it may also partition into brain tissue to produce beneficial actions. Pretreatment of animals with carvedilol limited ischemic injury in hippocampal CA1 regions after global ischemia/reperfusion injury

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[Lysko et al., 1992; Savitz et al., 2000]. Carvedilol also protected cultured neurons from excitotoxic and oxidative stress [Lysko et al., 1992, 1998].

There is evidence that carvedilol can modulate a variety of ion currents. For example, carvedilol blocked repolarizing K^+ currents and L-type Ca^{2+} currents in ventricular myocytes [Cheng et al., 1999; Karle et al., 2001]. The compound also stimulated proton leak and inhibited substrate oxidation in respiring mitochondria [Abreu et al., 2000; Oliveira et al., 2001]. Cloned K^+ channels can be modified by a variety of compounds that produce reactive oxygen species [Duprat et al., 1995]. However, little information is available regarding the underlying mechanism of actions of carvedilol on ion currents in neurons. It was also of interest to determine whether the effect of carvedilol on the ion channel is associated with its antioxidant effects.

The cholinergic neuroblastoma cell line NG108-15 cell has been widely used as a neuronal model in electrophysiology and pharmacology research [Brown and Higashida, 1988; Meves et al., 1999]. It displays neuronal characteristics and expresses Kv3.1a mRNA, and exhibits activity of delayed rectifier K^+ (K_{DR}) channels [Brown and Higashida, 1988; Yokoyama et al., 1989]. Kv3.1-Kv3.2 channels play an active role in neuronal excitability [Marom and Abbott, 1994; Hernandez-Pineda et al., 1999; Rudy et al., 1999]. The objective of the present study was to investigate whether carvedilol had any effects on ion currents and membrane potential in NG108-15 neuronal cells.

MATERIALS AND METHODS

Cell Culture

The clonal NG108-15 cell line was obtained from the European Collection of Cell Cultures (ECACC-88112302; Wiltshire, UK [Wu et al., 2001]). Cells were grown at 37°C in a humidified incubator with 5% CO_2/95% air and maintained at a density of 10^6/mL in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (vol/vol) and 2 mM L-glutamine. Experiments were performed after cells reached confluence (usually 5 to 7 days).

Electrophysiological Measurements

Immediately before each experiment, NG108-15 cells were dissociated and an aliquot of cell suspension was transferred to a recording chamber mounted on the stage of an inverted microscope (Diaphot-200; Nikon, Tokyo, Japan). The microscope was coupled to a digital video camera (DCR-TRV30; Sony, Tokyo, Japan) with magnification up to 1,500 × to monitor cell size during the experiments. Cells were bathed at room temperature (20–25°C) in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Patch pipettes were pulled from Kimax-51 glass capillaries (1.5–1.8 mm o.d., Kimble, Vineland, NJ) using a two-stage electrode puller (PB-7, Narishige, Tokyo, Japan), and the tips were fire-polished with a microforge (MF-83; Narishige). The pipette used had a resistance of 3-5 MΩ when immersed in normal Tyrode's solution. Ion currents were measured with glass pipettes in the whole-cell or cell-attached configuration of the patch-clamp technique, using an RK-400 (Biologic, Claix, France) or an Axopatch 200B patch-clamp amplifier (Axon Instruments, Union City, CA) [Hamill et al., 1981; Wu et al., 1999]. All potentials were corrected for liquid junction potential, a value that develops at the tip of the pipette when the composition of the pipette solution was different from that in the bath.

Data Recording and Analysis

The signals consisting of voltage and current tracings were displayed with a digital storage oscilloscope (Model 1602; Gould, Valley View, OH) and an LCD projector (AV600; Delta, Taipei, Taiwan). Ion currents were low-passed filtered at 1 kHz. A Digidata 1320A interface (Axon Instruments) was used for the analog-to-digital/digital-to-analog conversion. To reduce electrical noise, this interface device was connected to a Pentium III-based laptop computer (Slimnote VX_3; Lemel, Taipei, Taiwan) through a USB port, and then controlled with the Clampex subroutine in the pCLAMP 8.02 software (Axon Instruments). In order to establish a current-voltage (I–V) relationship for K^+ currents, voltage-activated currents during whole-cell experiments were analyzed using Clampfit subroutine (Axon Instruments), the Origin 6.0 software (Microcal Software, Inc., Northampton, MA), or custom-made macros in Excel (Microsoft, Redmond, WA).

To calculate percentage inhibition of carvedilol on $I_{K\,(DR)}$, each cell was depolarized from −50 to +50 mV with a duration of 300 msec. Current amplitudes measured at the end of each depolarizing pulse in the presence of carvedilol were compared with the control value. The concentration-dependent relationship of carvedilol on the inhibition of $I_{K\,(DR)}$ was fitted to a Hill function by nonlinear least-squares regression analysis with the Origin 6.0 software (Microcal Software Inc.), e.g.:

$$\text{Percentage inhibition} = (E_{max} \times [D]^{n_h}/(IC_{50}^{n_h} + [D]^{n_h}))$$

where [D] is the concentration of carvedilol; IC_{50} and n_h are the concentration required for a 50% inhibition and the Hill coefficient, respectively; and E_{max} is carvedilol-induced maximal inhibition of $I_{K\,(DR)}$.

Single-channel currents of delayed rectifier K^+ (K_{DR}) channels were analyzed with Fetch and Pstat subroutines in the pCLAMP software (Axon Instruments). Multi-gaussian adjustments of amplitude distributions between the channels were used to determine single-channel currents. The functional independence between channels was verified by comparing the observed stationary probabilities with the values of calculated according to the binomial law.

Values are provided as means ± s.e.m. with sample size (n) indicating the number of cells from which the data were obtained. The paired or unpaired Student's t-test and one-way analysis of variance with a least-significance difference method for multiple comparisons were used for the statistical evaluation of differences among means. A value of $P<0.05$ was considered to be statistically significant.

Simulation of Carvedilol-Induced $I_{K\ (DR)}$

To simulate the effect of carvedilol on $I_{K\ (DR)}$ in NG108-15 cells, a mathematical model similar to that described by Marom and Abbott [1994] was constructed. The following scheme shows the general form of this model.

$$C_0 \underset{\beta}{\overset{4\alpha}{\rightleftarrows}} C_1 \underset{2\beta}{\overset{3\alpha}{\rightleftarrows}} C_2 \underset{3\beta}{\overset{2\alpha}{\rightleftarrows}} C_3 \underset{4\beta}{\overset{\alpha}{\rightleftarrows}} O^* \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} OB$$

In this scheme, the channel opens by passing through a sequence of four closed states (C_0–C_3), ultimately reaching the open state O^*. OB represents the open-blocked states. Transitions between the sequences of states in the activation pathway are voltage dependent and can be described by an activation variable n satisfying the differential equation

$$dn/dt = \alpha_n(V) \times (1 - n) - \beta_n(V) \times n$$

In the absence of carvedilol, the number of channels in the open state, O^* is proportional to n^4.The voltage-dependent rate functions α_n and β_n are determined from our experimental data, k_{+1} and k_{-1} are those for block and unblock by carvedilol, and [B] is the blocker (carvedilol) concentration.

The forward (α) and backward (β) rate constants used in the simulation of $I_{K\ (DR)}$ were:

$$\alpha_n = 0.003 \times (35 - V)/\{\exp[(35 - V)/30] - 1\},$$

$$\beta_n = 0.0048 \times \exp[(25 - V)/30]$$

To model carvedilol-induced block of $I_{K\ (DR)}$, an inactivation variable h was included and the macroscopic current was then expressed as $I_{K\ (DR)}=G_k \times n(V)^4 \times h \times (V - E_k)$. Here, G_k is the maximal conductance of $I_{K\ (DR)}$ and E_k is the K^+ equilibrium potential. However, because carvedilol-induced block presented in this study was presumably a state-dependent rather than voltage-dependent process, the form of the equation describing the inactivation variable was modified as follows: $dh/dt = k_{+1} \times (1-h) - k_{-1} \times n^4 \times h$, where k_{+1} and k_{-1} are voltage-independent rate constants for block and unblock by carvedilol.

Compounds and Solutions

Carvedilol((±)-1-(carbazol-4-yloxy)-3-[[2-(*o*-methoxyphenoxy)ethyl]amino]-2-propanol) was obtained from Boehringer Mannheim (Mannheim, Germany). Linopiridine, tetraethylammonium chloride, *t*-butyl hydroperoxide, diazoxide and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) were purchased from Sigma Chemical (St. Louis, MO). 5-Hydroxydecanoate sodium was obtained from Biomol (Plymouth Meeting, PA). Tetrodotoxin and iberiotoxin were purchased from Alomone Labs (Jerusalem, Israel), and dendrotoxin was from Calbiochem (La Jolla, CA). Cell culture supplies were obtained from Life Technologies (Grand Island, NY). All other chemicals were commercially available and of reagent grade. Twice-distilled water deionized through a Millipore-Q system (APS Water Services Inc., Van Nuys, CA) was used in all experiments. The composition of normal Tyrode's solution was as follows (in mM): NaCl 136.5, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.53, glucose 5.5 and HEPES-NaOH buffer 5.5 (pH 7.4). To record membrane potential or K^+ current, the patch pipettes were filled with the solution (in mM): K-aspartate 130, KCl 20, KH_2PO_4 1, $MgCl_2$ 1, EGTA 0.1, Na_2ATP 3, Na_2GTP 0.1 and HEPES-KOH buffer 5 (pH 7.2).

RESULTS

Effect of Carvedilol on Delayed Rectifier K^+ Current ($I_{K(DR)}$) in NG108-15 Cells

The whole–cell configuration of the patch-clamp technique was used to investigate the effect of carvedilol on ion currents in NG108-15 cells. To record K^+ outward currents, cells were bathed in Ca^{2+}-free Tyrode's solution containing tetrodotoxin (1 μM) and $CdCl_2$ (0.5 mM). As shown in Figure 1, when the cell was held at −50 mV and the depolarizing pulses from −40 to +80 mV in 10-mV increments were applied, a family of K^+ outward currents with little inactivation was elicited. These outward currents were previously identified as $I_{K(DR)}$ [Brown and Higashida, 1988; Wu et al., 2001]. Within 1 min of exposing the cells to carvedilol (10 μM), the amplitude of $I_{K(DR)}$ measured at the end of 300-msec depolarizing pulses was significantly reduced at potentials ranging from 0 to +80 mV. For example, when depolarizing pulses from −50 to +50 mV were applied, the presence of

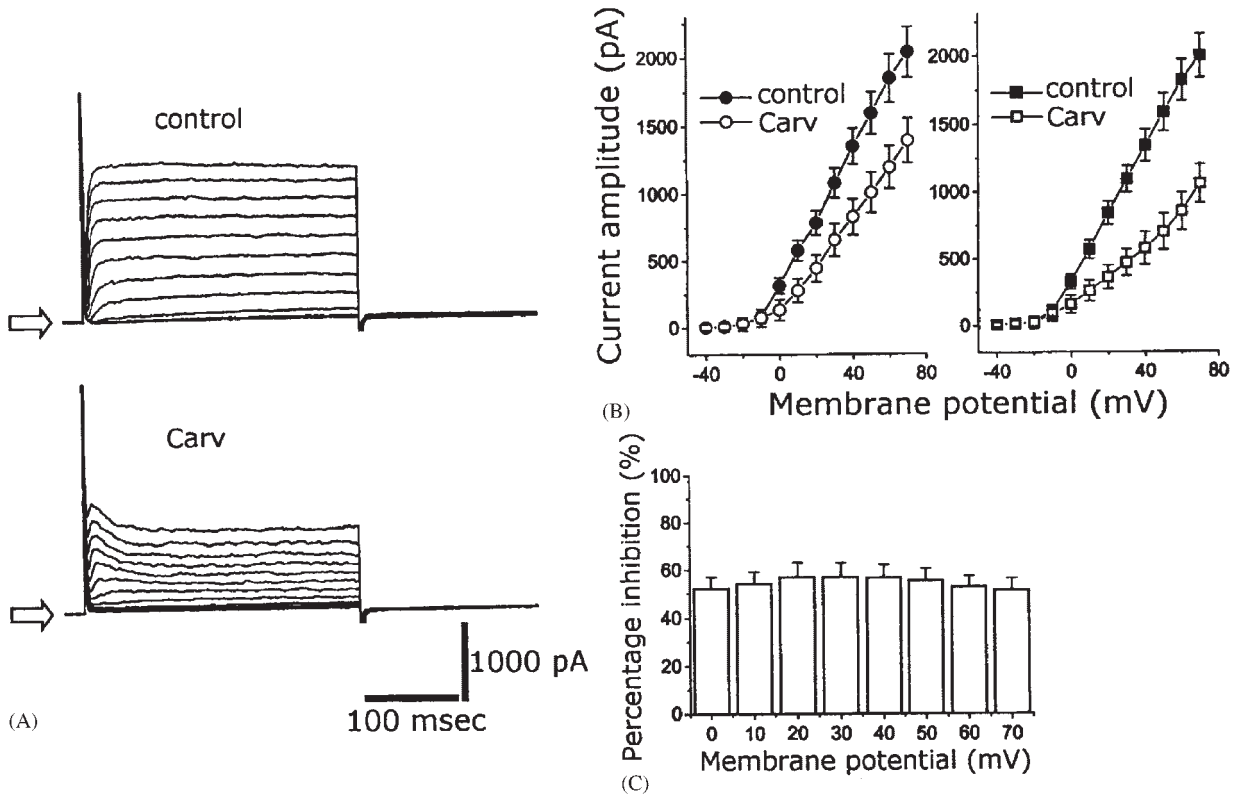

Fig. 1. Inhibitory effect of carvedilol on $I_{K\ (DR)}$ in NG108-15 cells. Cells were bathed in Ca^{2+}-free Tyrode's solution containing tetrodotoxin (1 μM) and $CdCl_2$ (0.5 mM). **(A)** Superimposed current traces in the absence and presence of carvedilol (10 μM). Cells were depolarized from −50 mV to various potentials ranging from −40 to +80 mV in 10-mV increments. Current traces shown in the upper panel are control, and those in the lower panel were obtained 1 min after addition of carvedilol (10 μM). Open arrows indicate the zero current level. **(B)** Averaged *I-V* relations for initial (left) and steady-state (right) components of $I_{K\ (DR)}$ in the absence (filled symbols) and presence (open symbols) of 10 μM carvedilol (Carv). Each point represents the mean ± s.e.m. (n = 8–11). **(C)** Bar graph showing little or no voltage-dependence for carvedilol-mediated inhibition of $I_{K(DR)}$. The percentage inhibition of $I_{K\ (DR)}$ by carvedilol (10 μM) was shown over the voltage range of 0 to + 70 mV (mean ± s.e.m.; n = 8–11 for each point).

carvedilol (10 μM) significantly decreased current amplitude at the end of the voltage pulses from 1,613 ± 141 to 742 ± 124 pA (n = 8). This inhibitory effect was readily reversed on washout of carvedilol.

Interestingly, a dramatic change in the time course of $I_{K(DR)}$ was also observed in the presence of carvedilol (Fig. 1A). These currents obtained after application of carvedilol activated a maximum and then decayed over time to a small fraction of their peak values at all voltages examined. However, there was no change in the early activation kinetics of these currents (i.e., *dI/dt*). Averaged *I-V* relations for the amplitude of initial and steady-state components of $I_{K(DR)}$ in the absence and presence of carvedilol (10 μM) are shown in Fig. 1B, indicating that carvedilol can reduce the amplitude of $I_{K(DR)}$ in a time-dependent manner.

The percentage inhibition of $I_{K(DR)}$ by carvedilol (10 μM) was also measured at test potentials between 0 and +70 mV. As shown in Fig. 1C, no significant difference was found in the percentage inhibition of carvedilol (10 μM) on $I_{K(DR)}$ across the voltages examined. For example, at a test potential of 0 mV, current was inhibited by 52 ± 5% (n = 6), while at +50 mV, inhibition was 54 ± 5% (n = 8). These data suggest that block by carvedilol of $I_{K(DR)}$ observed in NG108-15 cells exhibits little or no voltage dependence.

Figure 2A shows the relationship between the concentration of carvedilol and the percentage inhibition of $I_{K(DR)}$. Carvedilol (1–100 μM) suppressed the steady-state component of $I_{K(DR)}$ in a concentration-dependent manner. The IC_{50} value of carvedilol for the inhibition of $I_{K(DR)}$ was 5 μM, and 100 μM carvedilol could completely suppress the steady-state component of $I_{K(DR)}$. Thus, it is clear that carvedilol has a depressant effect on $I_{K(DR)}$ in NG108-15 cells.

Effects of Various K^+ Channel Blockers on $I_{K(DR)}$ in NG108-15 Cells

Effects of quinidine, dendrotoxin, iberiotoxin, 5-hydroxydecanoate sodium, and linopiridine on $I_{K(DR)}$ in

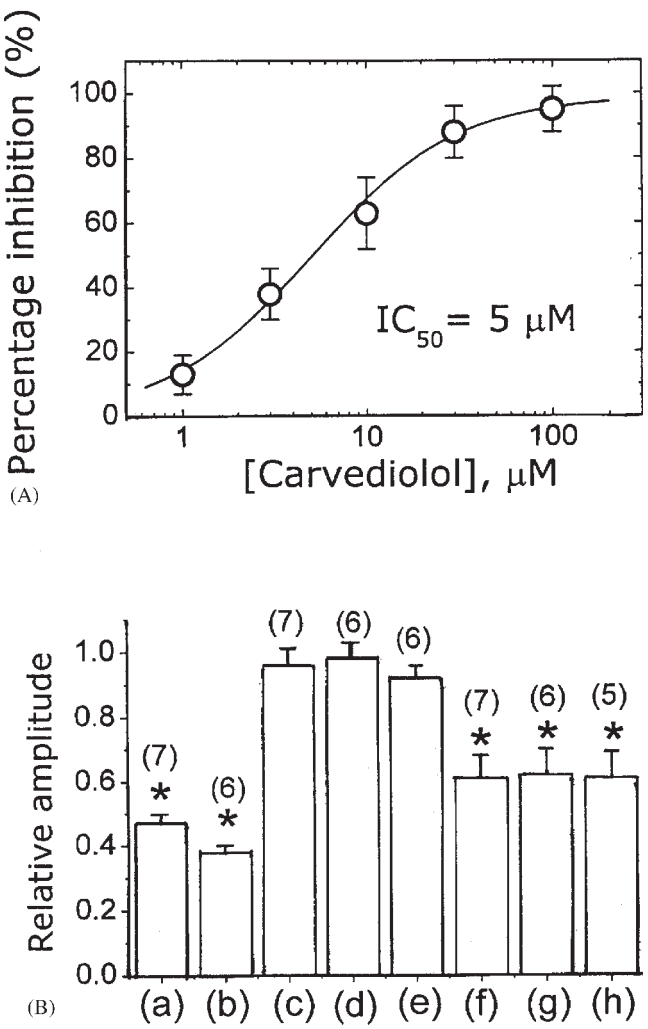

Fig. 2. Concentration-response curve for carvedilol-induced inhibition of $I_{K\ (DR)}$ **(A)** and effects of other related K^+ blockers on the amplitude of $I_{K\ (DR)}$ **(B)** in NG108-15 cells. Each cell was depolarized from −50 to +50 mV with a duration of 300 msec. In (A), the amplitude of $I_{K\ (DR)}$ obtained after addition of carvedilol was compared with the control value, i.e., in the absence of carvedilol (mean ± s.e.m.; n = 5–8 for each point). The smooth line represents the best fit to a Hill function. The values for IC_{50}, maximally inhibited percentage of $I_{K\ (DR)}$, and the Hill coefficient were 5 μM, 98%, and 1.1, respectively. In **(B)**, current amplitude measured at the end of voltage pulses in the control was considered to be 1.0 and the relative amplitude of $I_{K\ (DR)}$ after addition of each agent was compared and plotted. The parentheses denote the number of cells from which the data were obtained. a: quinidine (1 μM); b: dendrotoxin (1 μM); c: iberiotoxin (200 nM); d: 5-hydroxydecanoate sodium (10 μM); e: linopiridine (10 μM); f: carvedilol (10 μM); g: carvedilol (10 μM) plus *t*-butyl hydroperoxide (1 mM); h: carvedilol (10 μM) plus diazoxide (10 μM). Mean ± s.e.m. *Significantly different from control.

NG108-15 cells were also examined and compared. As shown in Figure 2B, other blockers of K^+ channels produced an inhibition of $I_{K(DR)}$. Quinidine (1 μM) and dendrotoxin (1 μM) decreased the amplitude of $I_{K(DR)}$ by approximately 55% and 60%, respectively. However, iberiotoxin (200 nM), 5-hydroxydecanoate sodium (10 μM), or linopiridine (10 μM) had no effect on $I_{K(DR)}$ (Fig. 2B). Iberiotoxin is a specific blocker of large-conductance Ca^{2+}-activated K^+ channels, and 5-hydroxydecanoate sodium is a mitochondrial ATP-sensitive K^+ channel blocker. Linopiridine was reported to block M-type K^+ current [Meves et al., 1999]. In addition, carvedilol–induced block of $I_{K(DR)}$ was not reversed by further application of *t*-butyl hydroperoxide or diazoxide (Fig. 2B). These results indicate that the observed $I_{K(DR)}$ in NG108-15 cells is sensitive to inhibition by quinidine and dendrotoxin, but not by iberiotoxin, 5-hydroxydecanoate sodium or linopiridine. Thus it appears that this inhibitory effect of carvedilol is not associated with the activity of ATP-sensitive K^+ channels or reactive oxygen species.

Effect of Carvedilol on $I_{K(DR)}$ in Cells Preincubated with *t*-Butyl Hydroperoxide, Ruthenium Red, or CCCP

Carvedilol is a lipophilic antioxidant compound [Tadolini and Franconi, 1998; Abreu et al., 2000] that also exerts potent antioxidative actions on mitochondrial membranes [Moser and Frishman, 1998; Abreu et al., 2000; Oliveira et al., 2001]. Previous reports have shown that reactive oxygen species could modify voltage-dependent K^+ channels [Duprat et al., 1995; Kourie, 1998]. For this reason, additional experiments were conducted to examine the effect of carvedilol on $I_{K(DR)}$ in cells treated with *t*-butyl hydroperoxide (1 mM), ruthenium red (30 μM) or CCCP (10 μM) for 30 min (Fig. 3). *t*-Butyl hydroperoxide is an oxidative agent, ruthenium red is a mitochondrial Ca^{2+} uniporter, and CCCP is a protonophore that breaks down the mitochondrial membrane potential required for Ca^{2+} uptake [Kourie, 1998; Li et al., 1998; Jan et al., 1999]. However, as depicted in Figure 3, in NG108-15 cells preincubated with *t*-butyl hydroperoxide (1 mM), ruthenium red (30 μM), or CCCP (10 μM) for 30 min, the inhibition effect of carvedilol (5 μM) on *I-V* relationship of $I_{K(DR)}$ remained unaltered. This lack of significant difference in the magnitude of carvedilol-induced inhibition on $I_{K(DR)}$ between control cells and cells treated with *t*-butyl hydroperoxide, ruthenium red, or CCCP indicates that the inhibitory effect of carvedilol on this current in these cells is unlikely to be due to its inhibition of oxidative activity.

Recovery from Block Induced by Carvedilol

To characterize carvedilol-induced block of $I_{K(DR)}$, recovery from block was further studied. In these experiments, a stimulation protocol consisting of a first (conditioning) depolarizing pulse sufficiently

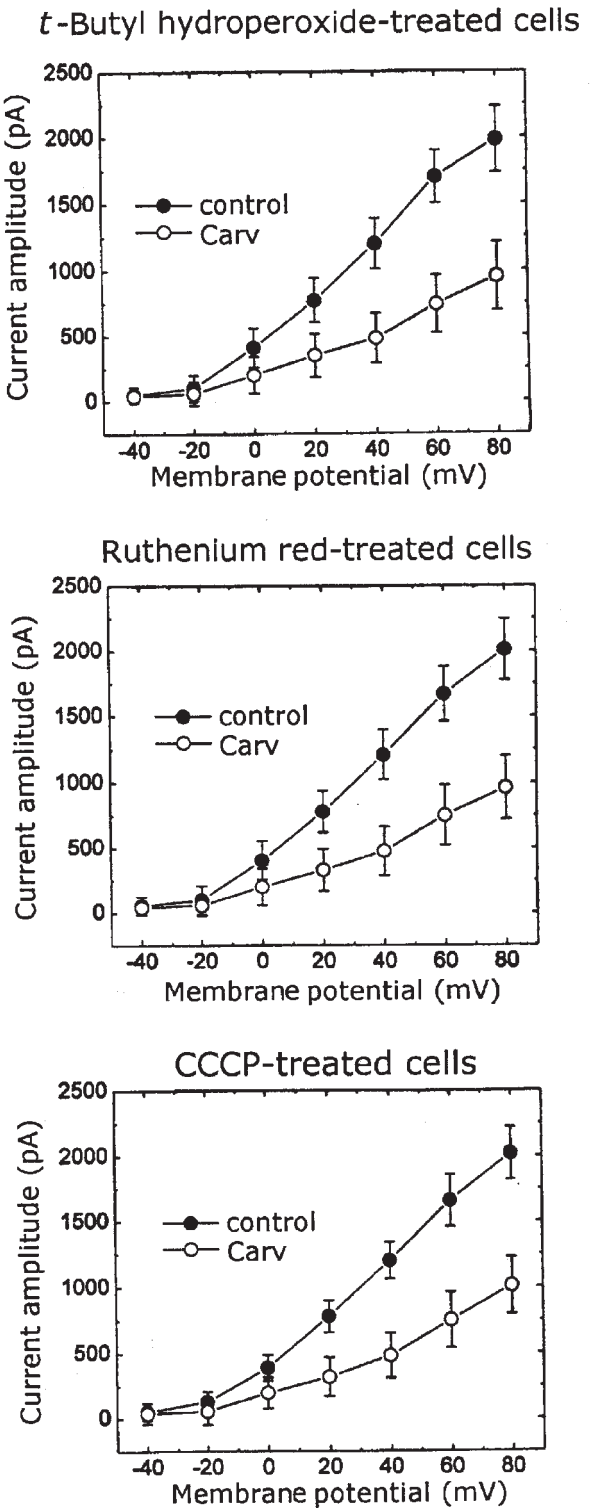

Fig. 3. Effect of carvedilol (10 μM) on the averaged *I-V* relations of $I_{K(DR)}$ in NG108-15 cells treated with *t*-butyl hydroperoxide, ruthenium red, or CCCP. Cells were preincubated with *t*-butyl hydroperoxide (1 mM), ruthenium red (30 μM), or CCCP (10 μM) for 30 min. Each cell was held at −50 mV and the depolarizing pulses from −40 to +80 mV in 20-mV increments were applied. Each point is the mean±s.e.m. (n = 4–8).

long to allow block to reach a steady state was used. During cell exposure to carvedilol (3 μM), the membrane potential was then taken to +50 mV from −50 mV for a variable time, after which a second depolarizing pulse (test pulse) was applied at the same potential as the conditioning pulse (Fig. 4). The ratios of the peak current amplitudes of $I_{K(DR)}$ evoked in response to the test and the conditioning pulse were then taken as a measure of recovery from block, and plotted versus interpulse interval. Recovery was generally complete, and its time course described by a single exponential with a time constant of 1.64± 0.25 sec (n = 5). In addition, the time constant of recovery was increased to 3.41 ± 0.56 sec (n = 5) during cell exposure to 10 μM of carvedilol, suggesting that the slowing of recovery caused by carvedilol may be due to open channel block.

Carvedilol-Induced Decay of Tail Current and Crossover

Assuming that carvedilol interacts with the open channel, it would be expected that the dissociation of this compound from the blocked channel might result in a transient conducting channel that subsequently closes over time. Figure 5 shows the superimposed currents evoked in response to the depolarizing pulse from −50 to +50 mV and the tail currents at −30 mV in the absence and presence of 3 μM carvedilol. In the absence of carvedilol, the depolarizing pulses activated $I_{K(DR)}$ that decayed upon repolarization to −30 mV. The presence of this compound was found to induce a decrease in current during the depolarizing pulse and peak amplitude of the tail current. Under control conditions, the tail current upon repolarization to −30 mV decayed with a time constant of 42.3 ± 3.8 msec (n = 6). However, after application of carvedilol (10 μM), deactivation of the tail current at −30 mV became biexponential decay with a time constant of 6.2 ± 1.1 and 92 ± 6.9 msec (n = 6). In addition, the time course of deactivating tail currents obtained in the presence of carvedilol (3μM) was found to be voltage sensitive. Thus, the tail current observed in the presence of carvedilol was found to exhibit two components. One component decayed faster than the tail current in the control, while the other declined more slowly. The slowing of the deactivating process induced by carvedilol produces a crossover phenomenon when the tail currents obtained under control conditions and in the presence of carvedilol. This phenomenon is consistent with the presence of a transient unblocking, thereby providing additional evidence for open channel block [Balser et al., 1991].

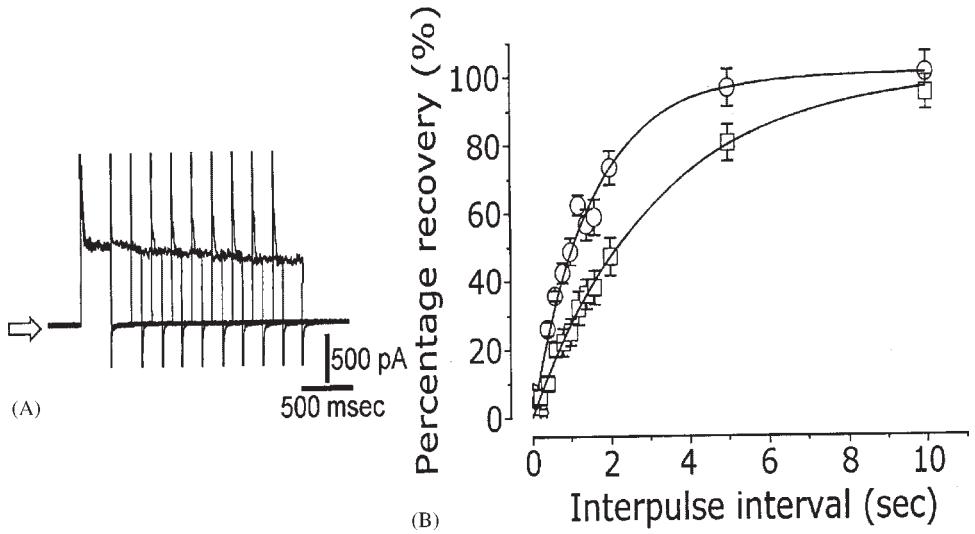

Fig. 4. Time course of the recovery of $I_{K\,(DR)}$ in the presence of 10 μM carvedilol. Cells, bathed in Ca^{2+}-free Tyrode's solution, were depolarized from −50 to +50 mV with a duration of 300 msec and different interpulse durations were applied. **(A)** Superimposed current traces in the presence of carvedilol (3 μM) obtained by a two-pulse protocol. Open arrow indicates the zero current level. **(B)** Time course of recovery from inactivation of $I_{K\,(DR)}$ caused by 3 μM (○) and 10 μM carvedilol (□). The time courses obtained in the presence of 3 and 10 μM carvedilol were well fitted by a single exponential with a value of 1.64 and 3.41 sec, respectively. Each point represents the mean ± s.e.m. (n = 4–8).

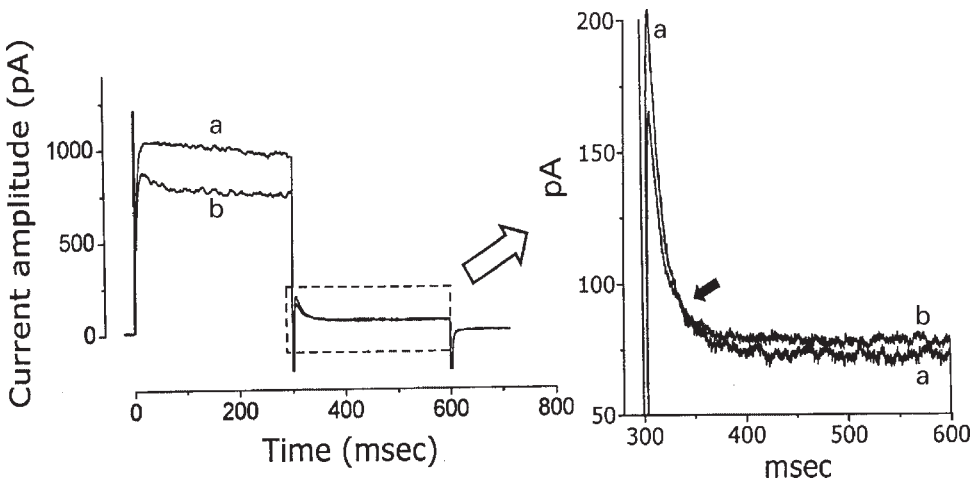

Fig. 5. Effect of carvedilol on the relaxation of tail current. The voltage pulses were depolarized from −50 to +50 mV and stepped back to −30 mV at which tail currents were measured. The tail current in control was found to decay more rapidly and cross over the tail current recorded in the presence of carvedilol (3 μM). The right panel shows current traces obtained in expanded scales. The arrow shown in right panel indicates the crossover phenomenon when voltage potential was returned to −30 mV. **(a)** control; **(b)** 10 μM carvedilol.

Kinetic Constants of Block by Carvedilol

Because in the presence of carvedilol, $I_{K(DR)}$ exhibited a pronounced peak followed by an exponential decay to a steady-state level, it was important to gain information about the kinetics of carvedilol-induced block of $I_{K(DR)}$. The concentration dependence of $I_{K(DR)}$ decay by carvedilol is illustrated in Figure 6A. Although the initial activation phase of $I_{K(DR)}$ was unchanged in the presence of carvedilol, its effects on $I_{K(DR)}$ resulted in a concentration-dependent increase in the rate of current decay and a decrease in the residual, steady-state current. In other words, increasing the concentration of carvedilol not only reduced the peak current, but also enhanced the apparent inactivation. Thus, the inhibitory effect of carvedilol on $I_{K(DR)}$ in NG108-15 cells can be explained by state-dependent blockers that bind to

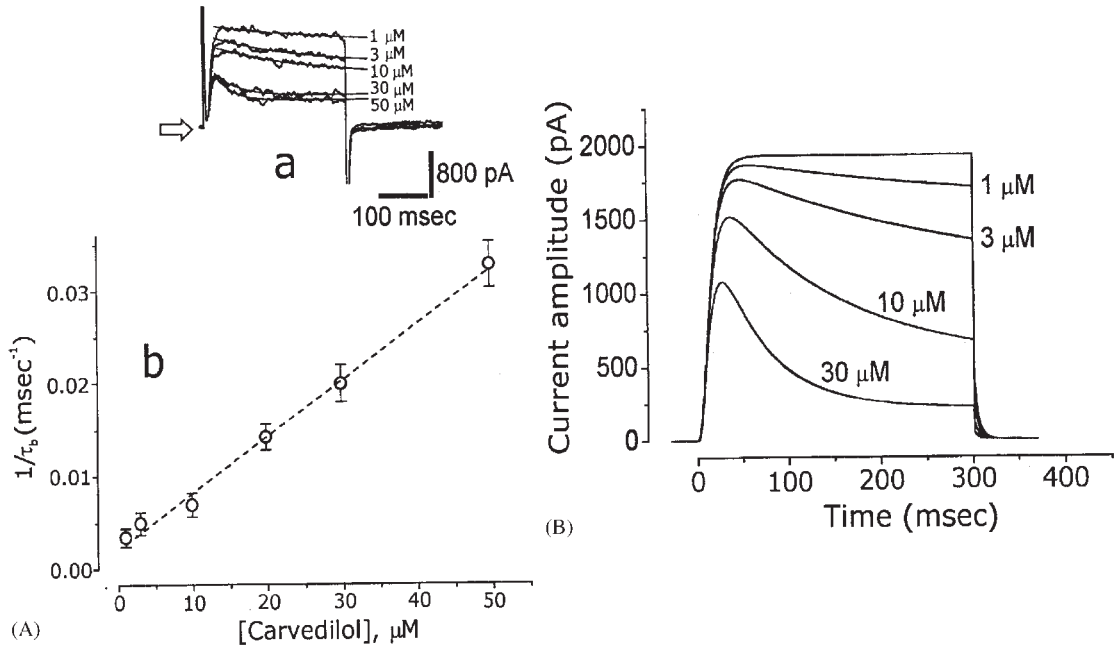

Fig. 6. Evaluation of the kinetics of carvedilol-induced block **(A)** and simulations of $I_{K\ (DR)}$ in the presence of various concentrations of carvedilol **(B)**. In (Aa), typical $I_{K\ (DR)}$ was elicited by depolarizing pulses to +50 mV in the presence of different concentrations of carvedilol in the bath. The time courses of current decay in the presence of 1, 3, 10, 30, and 50 μM carvedilol were fitted by a single exponential with a value of 278, 198, 172, 51, and 31 msec, respectively. Open arrow indicates the zero current level. In (Ab), the reciprocal of the time constant of the rate of block ($1/\tau_b$), obtained by a single-exponential fit of the decay phase of the current, was plotted versus the carvedilol concentration. Data points were fitted by a linear regression, indicating that block occurs with a molecularity of 1. Block (k_{+1}) and unblock (k_{-1}) rate constants, given by the slope and the *y*-axis intercept of the interpolated line, were 0.59 $msec^{-1}$ mM−1 and 0.0021 $msec^{-1}$, respectively. Each point represents the mean ± s.e.m. (n = 5–7). In (B), simulated control trace was generated with the assumption of typical values for $I_{K\ (DR)}$ in response to the depolarizing pulse from −50 to +50 mV described in Materials and Methods. For simulated currents in the presence of carvedilol, the unblocking rate constant, k_{-1}, was fixed at 0.0021 $msec^{-1}$ and the blocking rate constant, k_{+1} was expressed as the product of 0.00059 and the concentration of carvedilol in μM. The values shown at each current of panels Aa and B indicate the concentration of carvedilol used.

the open state of the channel according to the minimal kinetic scheme similar to that described by Armstrong [1969]:

$$C \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} O \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} O{\bullet}B$$

where α and β are the kinetic constants for the opening and closing of the channel, k_{+1} and k_{-1}, those for block and unblock by carvedilol, and [B] is the blocker (carvedilol) concentration. C, O, and O · B are the closed, open, and open-blocked states, respectively.

Block and unblock rate constants, k_{+1} and k_{-1}, were determined from the time constants of current decay evoked by the depolarizing pulses (Fig. 6A). Block and unblock rate constants could be estimated using the relation $1/\tau_b = (k_{+1} \cdot [B] + k_{-1})$ as previously described [Swenson, 1981]. In particular, k_{+1} and k_{-1}, respectively, result from the slope and from the *y*-axis intercept at [B] = 0 of the linear regression interpolating the reciprocal time constants (i.e., $1/\tau_b$) versus different carvedilol concentrations. As predicted by a first-order blocking scheme, the relationship between $1/\tau_b$ and [B] was linear with a correlation coefficient of 0.97 (Fig 6B), and the block and unblock rate constants obtained from five to nine different cells were calculated to be 0.59 $msec^{-1}$ mM^{-1} and 0.0021 $msec^{-1}$, respectively. Based on these rate constants, a value of 3.6 μM for the dissociation constant ($K_D = k_{-1}/k_{+1}$ could be derived. Notably, this value was found to agree well with the value of the carvedilol dissociation constant determined from the concentration-response curve (Fig. 2A). However, the rate constant of the inverse reaction (i.e., unblock rate constant), k_{-1}, showed little dependence on [B]; k_{-1} was 0.0014 ± 0.003 $msec^{-1}$ (n = 6) at 10 μM and 0.0015 ± 0.004 $msec^{-1}$ (n = 5) at 30 μM.

In order to do quantitative analysis of the effects of carvedilol on $I_{K(DR)}$ in NG108-15 cells, a mathematical model originally designed by Marom and Abbott [1994] was further applied. As shown in Materials and Methods, an inactivation model that is a voltage-

independent but state-dependent process was incorporated to the kinetic scheme. Importantly, this scheme provides a quantitative description of the $I_{K(DR)}$ obtained in the absence and presence of different concentrations of carvedilol in NG108-15 cells. Based on the rate constants determined from the fitting (Fig. 6A), simulated $I_{K(DR)}$ in the presence of different carvedilol concentrations were also obtained. Figure 6B summarizes the results of simulated $I_{K(DR)}$ evoked in response to a test potential to +50 mV from −50 mV with a duration of 300 msec in the absence and in the presence of different carvedilol concentrations (1, 3, 10, and 30 μM). The smooth lines shown in Fig. 6B denote convincing fits to the experimental results when numerical parameters were appropriately chosen. These characteristics are thus in good agreement with experimental results and supports the idea that a concentration-dependent increase by carvedilol in the rate of inactivation can account for the reduction of $I_{K(DR)}$.

Inhibitory Effect of Carvedilol on Single-Channel Current

The action of carvedilol was also examined at the single-channel level. Cell-attached configuration was performed in these experiments. Cells were bathed in Ca^{2+}-free Tyrode's solution. The presence of carvedilol in the pipette solution resulted in a reduction of channel activity as well as an acceleration in channel closing during the depolarizing pulses to + 50 mV from potential of −50 mV (Fig. 7). When carvedilol (10 μM) was included in the pipettes, the probability of channel openings was significantly decreased to 0.095 ± 0.009 from a control value of 0.198 ± 0.013 ($n = 6$). However, no significant change in the amplitude of single-channel current between the absence and presence of carvedilol was observed (2.1 ± 0.3 versus 2.0 ± 0.3 pA; $n = 6$). In addition, the mean open time of these channels in the presence of 5 μM carvedilol (54 ± 14 msec, $n = 5$) was significantly shorter than that in the control (89 ± 18 msec, $n = 5$). Thus, although the

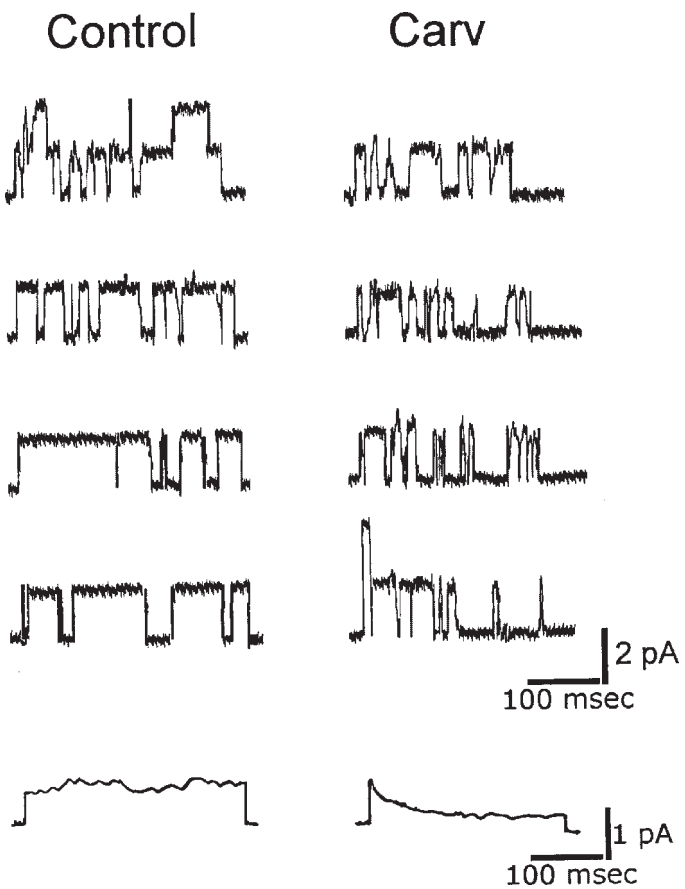

Fig. 7. Effect of carvedilol on single K_{DR} channel currents recording from cell-attached patches of NG108-15 cells. Single-channel currents were recorded during successive depolarization to +50 mV from a holding potential of −50 mV. Current traces shown on left side is the control, whereas those on right side is recorded with the pipette filled with carvedilol (10 μM; Carv). Channel openings are shown as upward deflection. The lowest trace represents the current average over 30 samples. Note that carvedilol decreases the probability of channel openings as well as accelerates channel closing; however; it does not modify single-channel amplitude.

presence of carvedilol did not affect the amplitude of single-channel current, it could decrease channel activity and inactivate channel opening.

Effect of Carvedilol on Action Potentials of NG108-15 Cells

In a final series of experiments, we examined the effect of carvedilol on changes in membrane potential. In these experiments, cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. Current-clamp configuration was performed with a K^+-containing pipette solution. The resting membrane potential and action potential duration in NG108-15 cells were -67 ± 8 mV and 162 ± 18 msec, respectively ($n = 25$). The action potentials of these cells were sensitive to extracellular Ca^{2+} and could be abolished by the presence of nimodipine (10 μM; [Wu et al., 2001]). The typical effect of carvedilol on action potentials in NG108-15 cells is illustrated in Fig. 8. Carvedilol (10 μM) significantly prolonged the action potential duration to 235 ± 21 msec from a control value of 163 ± 12 msec ($n = 14$). In addition, a slight reduction in both rising phase and amplitude of action potential could be due to the inhibitory effect of carvedilol on voltage-dependent Ca^{2+} current. However, the observed effect of carvedilol on spike broadening in NG108-15 cells could be mainly due to its inhibitory acions on $I_{K(DR)}$.

DISCUSSION

The results demonstrate that in these cells, carvedilol produces an inhibitory effect on delayed rectifier K^+ current [$I_{K(DR)}$] in a concentration- and state-dependent fashion. The major action of carvedilol on $I_{K(DR)}$ is thought to be predominantly through a state-dependent, open-channel block mechanism.

This study demonstrated that in NG108-15 neuronal cells, carvedilol induced a time-, concentration-, and state-dependent decay of $I_{K(DR)}$, without altering its activation kinetics. This observation, together with the good description of $I_{K(DR)}$ time course at different carvedilol concentrations with the computer simulation of the binding scheme, indicate that carvedilol may act as a state-dependent, open-channel blocker. Block by carvedilol of $I_{K(DR)}$ is of particular importance because it may have characteristics that make it significant from a pathophysiological point of view.

An important feature of the block of $I_{K(DR)}$ by carvedilol in NG108-15 cells was that the initial rising phase of the current (i.e., the activation time course) was unaffected. At the beginning of the voltage pulse, *dI/dt* will be proportional to the number of channels available for activation. The results showing that *dI/dt* was unchanged during cell exposure to carvedilol suggest that prior to channel activation, there should be the absence of any significant resting block of K_{DR} channels. Another noteworthy finding in this study is

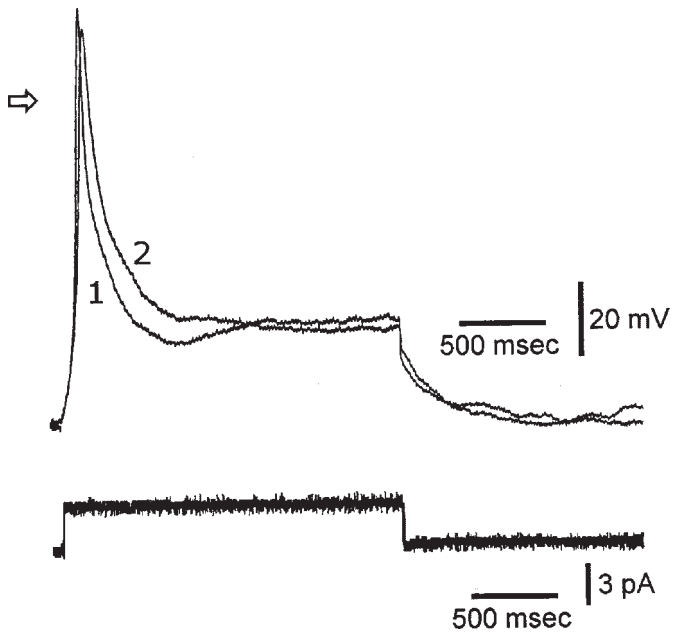

Fig. 8. Effect of carvedilol on action potentials of NG108-15 cells. Cells were bathed in normal Tyrode's solution containing 1.8 mM $CaCl_2$. The experiments were conducted under current-clamp conditions. Open arrow indicates the zero-potential level. Current-step protocol is shown in the lower part. (1) control; (2) carvedilol (10 μM). Notably, the presence of carvedilol can produce an increase in action potential duration.

that this compound tended to accelerate $I_{K(DR)}$ inactivation, suggesting that the carvedilol molecule appears to reach the blocking site only when the channel is in the open state. This feature can thus be incorporated in the minimal binding scheme, i.e., closed ↔ open ↔ open-blocked [Armstrong, 1969]. Inherent to this blocking scheme is that open-blocked channels are not closed unless carvedilol dissociates from the binding site, thus providing only one recovery path.

Block of $I_{K(DR)}$ by carvedilol is not instantaneous, but develops with time after the channels are opened, producing an apparent inactivation of the current. Tail currents of $I_{K(DR)}$ in the presence of carvedilol showed a blunted peak and a prolonged decay that is consistent with the possibility that the opening of channels was delayed by unbinding of the carvedilol molecule [Balser et al., 1991]. The blocking site of this compound appears to be located within the K^+ channel pore, only when the channel is open. However, the present finding showed a lack of effect of carvedilol on single-channel amplitude of K^+ channels, suggesting that the interaction of the channel with this compound seems to be secondary to alterations that are remote from the pore region of the channel. Moreover, because intracellular dialysis with carvedilol (100 μM) did not affect the rate of $I_{K(DR)}$ inactivation (data not shown), block by carvedilol of $I_{K(DR)}$ seen in NG108-15 cells may not occur via an intracellular site.

As shown in Figure 5, the deactivating tail current observed in the presence of carvedilol was found to cross over the tail current observed in the absence of drug when the two tracings were superimposed. This crossover suggests that carvedilol block can slow channel closing, and that this compound must be dissociated from the channel before the channel can be activated [Balser et al., 1991]. Therefore, upon repolarization in the presence of carvedilol, the open state unblocks and then either closes, or binds the compound again to repeat the process, thus leading to a current increase followed by a slowing of the rate of current decay and the observed crossover.

It is noteworthy that the time course of the tail currents obtained in the presence of carvedilol was voltage sensitive. The tail current in controls decayed more rapidly and crossed over the tail current recorded in the presence of carvedilol. The mean open time of single-channel current was also decreased in the presence of carvedilol. Taken together, these results suggest that the antagonism of $I_{K(DR)}$ by carvedilol might result primarily from open channel block. Indeed, the inhibition produced by carvedilol in this study is similar to that reported for human *ether-à-go-go* related gene (HERG) K^+ channels expressed in *Xenopus* oocytes in a open-state manner [Karle et al., 2001]. However, it still remains to be clarified to what extent carvedilol may have a higher affinity for the open conformation of K^+ channels than the closed conformation without evoking open channel block. In this study, a mathematical model was also designed to describe carvedilol-induced block of $I_{K(DR)}$ in terms of modified Hodgkin and Huxley formalism [Marom and Abbott, 1994]. This simulation model was found to be useful for the study of carvedilol-induced block on $I_{K(DR)}$ in NG108-15 cells.

The observed effects of carvedilol on $I_{K(DR)}$ in NG108-15 cells clearly did not involve the suppression of either Ca^{2+}-dependent K^+ channels or ATP-sensitive K^+ channels. All recordings of $I_{K(DR)}$ presented here were conducted in a Ca^{2+}-free Tyrode's solution containing $CdCl_2$ (0.5 mM). Furthermore, neither peak amplitudes nor inactivation kinetics of $I_{K(DR)}$ were altered by iberiotoxin or 5-hydroxydecanoate sodium. However, the blockade of $I_{K(DR)}$ by carvedilol could be responsible for its actions on the broadening of action potentials [Wang et al., 1998].

Because the IC_{50} value required for carvedilol-induced block of $I_{K(DR)}$ in the present study is quite close to its therapeutic concentrations, it might be expected that there would be a link between antioxidative property and its inhibitory effect on $I_{K(DR)}$ [Tadolini and Franconi, 1998; Abreu et al., 2000]. Indeed, it has been reported previously that increased reactive oxygen species can modify the activity of voltage-dependent K^+ channels [Duprat et al., 1995; Kourie, 1998]. However, the present experiments showed that in NG108-15 cells preincubated with *t*-butyl hydroperoxide, ruthenium red, or CCCP, carvedilol was also found to suppress the amplitude of $I_{K(DR)}$ effectively. Thus, it would seem unlikely that carvedilol-mediated inhibition of this current presented here is due to its effect on antioxidant property. Accordingly, it appeared that carvedilol did not bind to α- or β-adrenoceptors in these cells.

Carvedilol may also be a valuable tool for probing the structure and function of K^+ channels from the K_{DR} family, because the pore region of the channel protein to which it binds is of particular relevance for open-channel blockade [Durell and Guy, 1992]. The role of K_{DR} channels, particularly at the members of the Kv3 superfamily present in time-coding neurons, is to stabilize the resting membrane potential and effectively reduce the width of action potentials [Wang et al., 1998; Rudy et al., 1999; Deuchars et al., 2001; Li et al., 2001; Macica and Kaczmarek, 2001; Parameshwaran et al., 2001]. The blockade of K_{DR} channels by carvedilol, together with an increase in action potential duration, may be responsible for its

effects on neuronal excitability [Durell and Guy, 1992; Rudy et al., 1999; Krishtal et al., 2001]. Because recovery from inactivation in the presence of carvedilol observed in this study was slow, more than 5 sec was required for the channel to recover completely. Consequently, carvedilol-induced block of $I_{K(DR)}$ will even become very significant when a train of action potentials occurs, because of the fact that under these conditions, the availability of K_{DR} channels is decreased as a function of firing frequency [Erisir et al., 1999]. More importantly, it remains to be determined whether this blocking action is related to carvedilol-induced inhibition of proliferation and migration in vascular smooth muscle cells [Ohlstein et al., 1993] or neuroprotection in cerebral ischemia [Lysko et al., 1992; Savitz et al., 2000].

Block of $I_{K(DR)}$ induced by carvedilol also has features that are interesting from the molecular-biophysical point of view. For example, previous work has demonstrated that a glutamate residue (E-418) located at the external end of segment S5 appeared to be significant for the binding of K^+ ions and maintenance of the channel open conformation in Shaker K^+ channels [Ortega-Saenz et al., 2000]. Mutations of amino acids in the pore (positions 447 and 449) were also reported to affect K^+ binding and channel inactivation [Molina et al., 1998]. It thus remains to be determined whether this residue is important for the binding of carvedilol to the external entry of the channel, or whether carvedilol can compete with K^+ for the same site in the K_{DR} channel present in NG108-15 cells.

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